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Antimicrobial proteins from cowpea root exudates: inhibitory activity against *Fusarium oxysporum* and purification of a chitinase-like protein

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Abstract

Plants exude a variety of substances through their external surfaces and from germinating seeds, some of which have an inhibitory action against plant pathogens. The aim of this study was the investigation and characterization of defense proteins present in exudates from roots of cowpea seedlings (*Vigna unguiculata* (L.) Walp.). Root exudates were collected from seedlings that were grown hydroponically in three different media, including, 100 mM sodium acetate buffer pH 4.5, water pH 6.0 and 100 mM sodium phosphate buffer pH 7.5. The proteins from these exudates were analyzed by SDS–PAGE and SDS–Tricine–PAGE and the presence of antimicrobial proteins in the exudates was investigated by immunological and enzymatic assays. Results showed that roots from cowpea seedlings contained β-1,3-Glucanases, chitinases and lipid transfer proteins (LTPs), all of which may potentially function as plant defense proteins. Immunolocalization of one of these proteins, chitinase, revealed its presence in the xylem cell wall vessel elements. These exudates also demonstrated an inhibitory effect on the growth of the fungus, *Fusarium oxysporum, in vitro*. The results suggest that plant roots may exude a variety of proteins that may function to repress the growth of root pathogenic fungi.

Introduction

Cowpea (*Vigna unguiculata*) is a tropical legume that originated in Africa and is now cultivated in most tropical regions of the world (Singh and Rachie, 1985). In Brazil, cowpea is mostly cultivated in the northeastern region of the country and is the main protein source for the majority of the poor population of this region. Cowpea seeds are heavily attacked both in the field and in storehouses by the cowpea weevil (*Callosobruchus maculatus*) and is also affected by a great number of pathogens. It is thought that this high susceptibility to predation is associated with low levels of defenses, either constitutive or induced, that are presented by this plant (Gomes and Xavier-Filho, 1994; Xavier-Filho et al., 1989). Nevertheless, several cowpea varieties or cultivars are able to express defense responses against many viruses, bacteria, fungi and insects (May et al., 1988). Many of these defenses are proteinaceous in nature (Carvalho et al., 2001; Gomes et al., 1996; Sales et al., 2000; Xavier-Filho, 1991) and are commonly found as part of a general defense response present in plant seeds.

Plants exude a variety of substances through their cell surfaces of both the above and below ground plant parts, some of which possess an

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inhibitory action against plant pathogens (Agrios, 1997). Furthermore, a number of these compounds have beneficial effects on germination whereas others inhibit seed colonisation by predators (Barbour et al., 1991). In addition, several preformed plant proteins have been shown to inhibit hydrolytic enzymes that are involved in host cell wall degradation by pathogens, and to inhibit proteinases, inactivate foreign ribosomes, or to increase the permeability of fungal plasma membranes (Shewry and Lucas, 1997).

Through the exudation of a wide variety of compounds, roots affect the soil microbial community in their immediate vicinity, influence resistance to pests, support beneficial symbioses, alter the chemical and physical properties of the soil, and inhibit the growth of competing plant species (Bertin et al., 2003). In earlier research, Netzly and Butler (1986) demonstrated the presence of proteins, lipids, amino acids, quinones and phenolics in sorghum root exudates, some of which may potentially affect microorganisms. Other studies also have shown the presence of different flavonoides and hydroquinones in root exudates (Hallak et al., 1999; Isobe et al., 2001). Very little, however, has been reported with regard to antimicrobial defense proteins that may occur in root exudates. In this report, we describe the identification and characterization of antimicrobial proteins exuded from cowpea roots seedlings, the isolation of a chitinase-like protein, and examine the inhibitory activity of these exudates against the fungus, *Fusarium oxysporum*.

Material and methods

Biological material

Cowpea (*Vigna unguiculata* L. Walp.) seeds of the Epace-10 cultivar, which are susceptible to the bruchid insect *Callosobruchus maculatus* (Xavier-Filho et al., 1989), were supplied by the Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil where they were developed. The fungus *Fusarium oxysporum* (agent of wilting, a disease of many plants), was kindly supplied by CNPAF/EMBRAPA, Goiania, Goiás, Brazil. The fungus was maintained on agar Sabouraud (1.0% peptone, 2.0% glucose and 1.7% agar-agar). For the preparation of *F. oxysporum* conidia, fungal cultures were transferred to Petri dishes containing Sabouraud agar for 12 days. After this period, 10 mL sterile saline was poured on the dishes and then the mycelium was gently agitated with a Drigalski loop for 1 min for conidia liberation. Conidia were quantified in a Neubauer chamber and their concentration was adjusted to appropriate dilutions.

Recovery of the root exuded proteins

Surface sterilized seeds were dispersed on moistened filter paper and maintained in the dark for 5 d, until germination took place. After this period, the seedlings were transferred to sterilized 250-mL Erlenmyer flasks where the roots were submerged in media consisting of 100 ml of 100 mM sodium acetate buffer pH 4.5, water pH 6.0, or 100 mM sodium phosphate buffer pH 7.5. The flasks were kept on a rotary shaker for 12 h at 70 rpm (Innova 4300 incubator, New Brunswick Scientific). After this period, the seedlings were carefully removed from the flasks, and the contents of the flask were filtered, precipitated with ammonium sulfate (0–90%), after which the precipitates were dialyzed against distilled water and lyophilized. The recovered powder was stored at −20 °C until use in experiments. Protein content was determined as described by Bradford (1976) using BSA as protein standard.

Protein purification

Defense proteins with affinity to chitin were purified using affinity chromatography. Chitin was prepared from powdered lobster shells according to the method of Hackman and Goldberg (1964). The chitin column (1.5 × 5 cm) was previously equilibrated with 0.05 M sodium acetate buffer (pH 4.0). Five milligrams of total exudates, obtained with acid medium (acetate buffer, pH 4.5), were then mixed with 1 mL of equilibrium buffer and loaded into the column. Chromatography was initially run with equilibrium buffer, followed by 0.1 M phosphate buffer, pH 1.5, and a final elution with 0.1 M HCl. Fractions of 1 mL, corresponding to protein peaks, were combined and subjected to dialysis and lyophylization.

Gel electrophoresis

SDS–PAGE was carried out according to the denaturing method of Laemmli (1970) on 10% gels. SDS–Tricine–PAGE was performed according to the method of Schagger and Von Jagow (1987) on 16% gels.

Antibodies and Western blotting

Polyclonal antibodies against a purified lipid transfer protein (LTP) from cowpea seeds were prepared as described by Carvalho et al. (2001). Antiserum against a purified chitinase from *Adenanthera pavonina* seeds was prepared as described by Santos et al. (2004). Western blotting was performed by transferring proteins to nitrocellulose membranes after PAGE, according to the method described by Towbin et al. (1979). The blots were incubated overnight with the primary antiserum against chitinase and LTP (1:2000) in blocking buffer (0.1 M phosphate buffer plus 0.15 M NaCl, pH 7.3, containing 2% defatted milk). After incubation, the membranes were washed 4 times, 10 min each time, with a 0.1 M phosphate buffer containing 0.15 M NaCl, pH 7.3. The blots were incubated with goat anti-rabbit immunoglobulin conjugated with peroxidase (Sigma Immuno Chemicals) (1:2000) in blocking buffer for 2 h at room temperature and were then washed as above. Blots were developed using a chemiluminescence detection Kit (ECL-Western blotting detection reagents/RPN 2209), according to the manufacturer's instructions. The chemiluminescence signal was recorded on an X-ray film and photographed.

Enzyme assays

β-1,3-Glucanase was assayed by the method of Fink et al. (1988), which employs laminarin (Sigma Chemicals Co., St. Louis) as a substrate. Exudates (20 μL of sample from 2 mg mL^{-1} solution) were incubated at 37 °C, for 6 h, with the substrate (0.25 mL of 2 mg mL^{-1} laminarin solution in 0.05 M acetate buffer, pH 5.0) in a final volume of 0.5 mL. Color was developed by the copper-arsenomolybdate method (Nelson, 1944; Somogyi, 1952) and the absorbance was read at 500 nm. One unit of activity was defined as the concentration of enzyme that produced an absorbance value of 0.001.

Chitinase activity was determined by the fluorescence released after cleavage of the fluorogenic substrate, 4-methylumbelliferyl-β-D-N, N′, N″-triacetylchitotrioside (Sigma Chemical Co.). The reactions were performed by incubating 0.25 mM substrate with 20 μL of each exudate (1 mg mL^{-1} solution) in 2 mL of 25 mM phosphate buffer (pH 5.0) and the liberated methylumbelliferyl (MU) was measured fluorimetrically employing 320 nm primary and 460 nm secondary filters in a Hitachi F4500 fluorescence spectrophotometer (O'Brien and Colwell, 1987). A calibration curve was constructed using methylumbeliferone-sodium salt. One unit of enzyme activity was defined as 1 nmol min^{-1} liberation of methylumbeliferone.

Antifungal activity assay

The effect of root exudates on the growth of *F. oxysporum* was assayed as described by Breokaert et al., 1990 with modifications. The conidia (20,000 cells mL^{-1} in 1 mL of saline solution) were incubated at 28 °C in 200 μl microplates followed by the addition of the exudates at a final concentration of 100 μg mL^{-1}. Controls without addition of root exudates were also utilized. Optical readings at 620 nm were taken at zero time and every 6 h for the following 60 h. These readings were taken against a blank containing only the culture medium. After the 60 h growth period, cells were separated from the growth medium by centrifugation at 2.000 *g* for 5 min, washed in 0.1 M Tris–HCl (pH 8.0) and plated for observation on an optical microscope at 400 × magnifications. All the experiments were run in triplicate.

Tissue localization of chitinase

To localize chitinase activity in the plant root tissues, roots that had been used for exudate collection were sectioned and fixed for 2 h in a 0.05 M cacodylate buffer, pH 7.0, containing 0.1% glutaraldehyde (v/v) and 4% paraformaldehyde (v/v). The samples were rinsed 3 times in 0.05 M cacodylate buffer, pH 7.2, dehydrated in solutions of increasing concentrations of methanol (30 – 90%, v/v) and were then processed for LR Gold

embedding. Sections (0.8–1.0 μm thick) were placed on to glass slides and subjected to the tissue localization assay. The sections were immunolabelled by treating the slides as follows: (a) ammonium chlorate (50 mM, pH 5.3), 30 min; (b) PBS + BSA (10 mM phosphate buffer, 0.15 M NaCl, pH 7.3 with 1% BSA), 20 min; (c) pre-immune serum (1:100) in PBS + BSA, 20 min; (d) anti-chitinase serum (1:25) in PBS + BSA, 2 h at room temperature; (e) six changes of PBS + BSA, 10 min each, (f) goat anti-rabbit IgG antibody coupled with 10 nm colloidal gold (Sigma) (1:50) in PBS + BSA, 2 h at room temperature; and (g) ten changes of PBS + BSA, 6 min each. Immunolabelling was visualized by a silver intensification kit (IntenSEe Silver Enhancement Kit, Amersham, RPN 491, Buckinghamshire, UK), following the manufacturer's instructions. The reaction was visualized by optical microscopy via the deposition of a precipitate on gold colloidal particles coupled with a secondary antibody. Slides were counter-stained with Toluidene Blue.

Results and discussion

Identification of the exuded proteins by electrophoresis

To identify proteins in exudates obtained from cowpea seedling roots, different soaking media were used that varied with respect to their ion composition and pH. Proteins in the exudates were first analyzed by SDS–PAGE and SDS–Tricine–PAGE. This analysis revealed that many proteins were exuded from imbibed cowpea seedling roots (Figure 1). These results suggest a state of intense outflow of proteins from the roots during the onset of germination. SDS–PAGE demonstrated significant differences in the protein patterns of seed exudates obtained for the different media (Figure 1a). In acetate buffer (pH 4.5), the released proteins had molecular masses ranging from 14 to 97 kDa, with the most abundant peptides having molecular masses of around 14 kDa (Figure 1a, line 1). With water (pH 6), few proteins were obtained in comparison with the other two media and with phosphate buffer (pH 7.5) the predominant proteins ranged from 14 to 97 kDa (Figure 1a, lines 2 and 3), but there was fewer low molecular weight proteins and more high molecular weight proteins as compared with the proteins that were obtained in acetate buffer (Figure 1a). SDS–Tricine–PAGE gave a better separation for root exuded proteins with low molecular masses ranging from 8 to 25 kDa. As with SDS–PAGE, there were differences between the protein patterns of root exudates obtained for the different media, in which there was enrichment of low molecular weight proteins as compared with those obtained from acetate and water (Figure 1b). These results suggest that exuded proteins from cowpea roots are differentially expressed and/or extractable according to the environmental conditions and that the composition varies in relation to the pH and

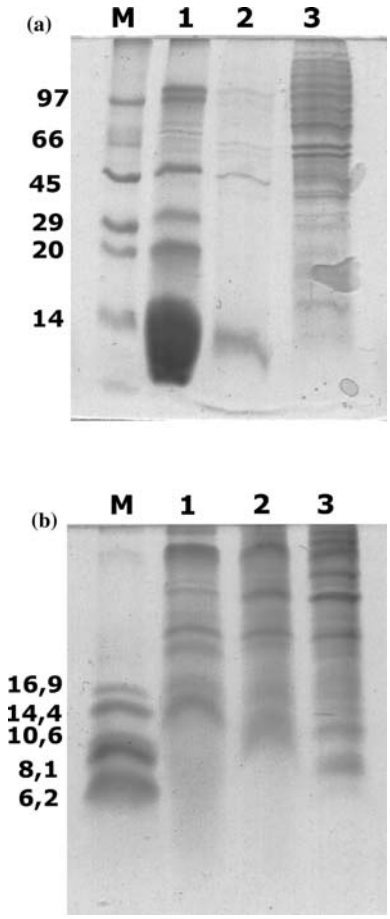

Figure 1. SDS–PAGE electrophoresis (a) and SDS–Tricine–PAGE gel electrophoresis (b) of root exudates obtained after 12 h in (1) acetate buffer, pH 4.5, (2) water, pH 6.0, and (3) phosphate buffer, pH 7.5 (3). Lane (M) provides values for molecular mass (kDa) markers.

ionic composition of the root environment (Bertin et al. 2003).

Characterization of the exuded proteins

Plants produce enzymes such as β-1,3-Glucanases and chitinases (Bruner et al., 1998; Recorbet et al., 1998) that can break down pathogen cell wall components (Carlile et al., 2001). It has become increasingly clear that such proteins play an important role in the protection of plants. (Arlorio et al., 1992; Ji and Kuć, 1996; Vannini et al., 1999). Another group of antimicrobial proteins are the basic and cysteine-rich peptides. These peptides are thought to play a role in the protection of plants against microbial infections; an example of this group being the lipid transfer proteins (LTP) (Kader, 1996). A defense role for these peptides is supported by their preferential cell-wall location in epidermal cells throughout the plant and by the increased expression of the genes that encode these peptides in response to pathogens (Carvalho et al., 2001; Jung et al., 2003; Pyee et al., 1994; Regente and De La Canal, 2000). In the research reported here, the presence of defense proteins such as LTPs, chitinases and β-1,3-Glucanase in exudates obtained from imbibed roots was investigated using Western blotting and specific enzyme assays. The presence of chitinases (Figure 2a), LTPs (Figure 2b) and β-1,3-Glucanase (Table 1) were detected by the combined use of specific anti-sera and enzymatic activities. These three defense proteins were present in all samples obtained after first 12 h of seed imbibition. This indicates that antimicrobial proteins are released from roots during early plant development. Western blotting for chitinases showed no significant quantitative differences between the three exudates analyzed. The opposite seems to be the case for LTP, since this protein was detected only in root exudate at high pH in the medium containing phosphate buffer at pH 7.5. In contrast, β-1,3-Glucanase assays revealed high specific activity of this enzyme in exudates at low pH in acetate buffer (pH 4.5) and water (pH 6.0) (Table 1). Barbour et al. (1991) demonstrated that, during germination, root seedlings release several compounds that may interfere with the development of microorganisms, particularly chemical inhibitors that prevent seed colonization by predators. These compounds, however, have not been characterized. In addition, several preformed plant proteins from roots have been reported to be present in plants and can act as inhibitors of various pathogens (Burketova et al, 2003; Recobert et al., 1998; Lam and Ng, 2001).

Table 1. β −1,3-glucanases activity found in the root exudates

Exudation medium	Units.mg^{-1} of protein
Sodium actetate buffer, pH 4.5	380,0
Water, pH 6.0	412,5
Sodium phosphate buffer, pH 7.5	20,0

Enzyme was measured in the freeze-dried crude exudate. Results are the average of three independent determinations

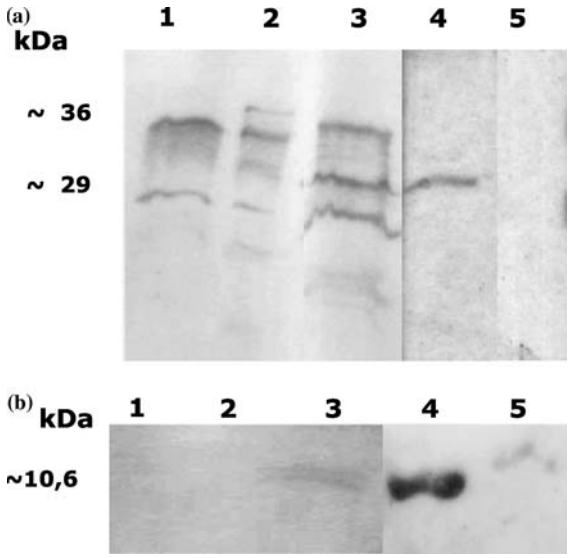

Figure 2. Western blotting of different exudates from imbibed seed roots using anti-sera against chitinase (a) and LTP (b) obtained after 12 h in (1) acetate buffer, pH 4.5, (2) water, pH 6.0, and (3) phosphate buffer, pH 7.5; (4) positive control using chitinase isolated from *Adenanthera pavonina* seeds and LTP isolated from *Vigna unguiculata* seeds; (5) negative control was prepared by replacing the specific proteins by buffer sample.

Purification of a chitinase-like protein from cowpea root exudates

Chitinases have been purified and/or cloned from many plants. Here, the purification of a protein with chitinase characteristics from root exudates was achieved through the utilization of chitin affinity chromatography. Using root exudates

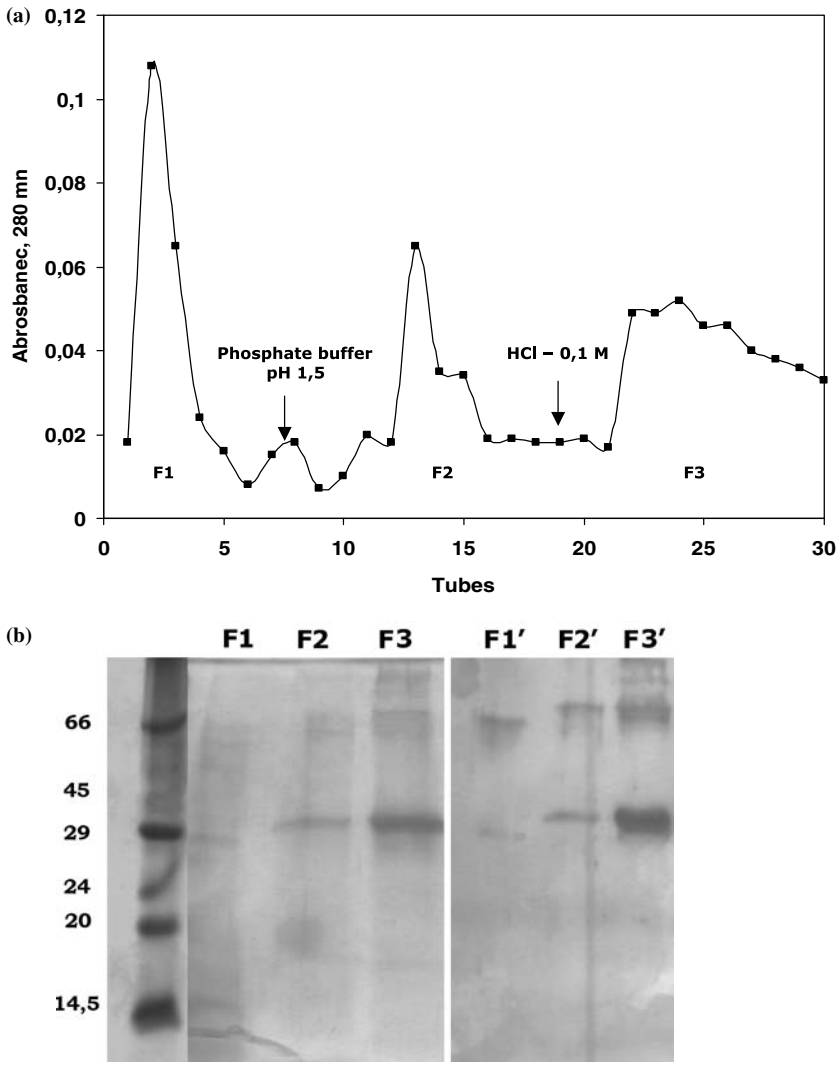

Figure 3. (a) Purification of the chitinase in exudate from cowpea seedling root using affinity chromatography (b) SDS–PAGE electrophoresis (F1, F2 and F3) and Western blot (F1′, F2′ and F3′) of proteins of the fractions obtained after chitin affinity chromatography using a specific anti-IgG chitinase.

from plant roots that were placed in acetate buffer (pH 4.5), three peaks were obtained, designated here as F1 (non-retained and eluted in the column equilibrium buffer), F2 (retained and eluted with phosphate buffer, pH 1.5) and F3 (retained and eluted with HCl 0.1 M) (Figure 3). Chitinase containing fractions were identified by Western blotting using a specific antibody raised against a chitinase from *A. pavonina* seeds. PAGE of these peaks (F1, F2 and F3) gave a major band on the gel, with a molecular mass of 30 kDa. (Figure 3a). To confirm that these proteins were chitinases, we also analyzed the effect of a specific anti-serum against chitinase on the exudate in medium with acetate. This analysis showed the presence of a major protein with a molecular mass of about 30 kDa, identical to the major protein visualized in electrophoresis for all exudates. However, a strong reaction was only visualized in the F3 peaks, which is most strongly bound to the chitin column, as compared with F1 and F2 (Figure 3b). The molecular mass for the identified chitinase falls within the range of molecular weights that has been previously reported for chitinases (Allona et al., 1996; Gijzen et al., 2001; Leah et al, 1991). This

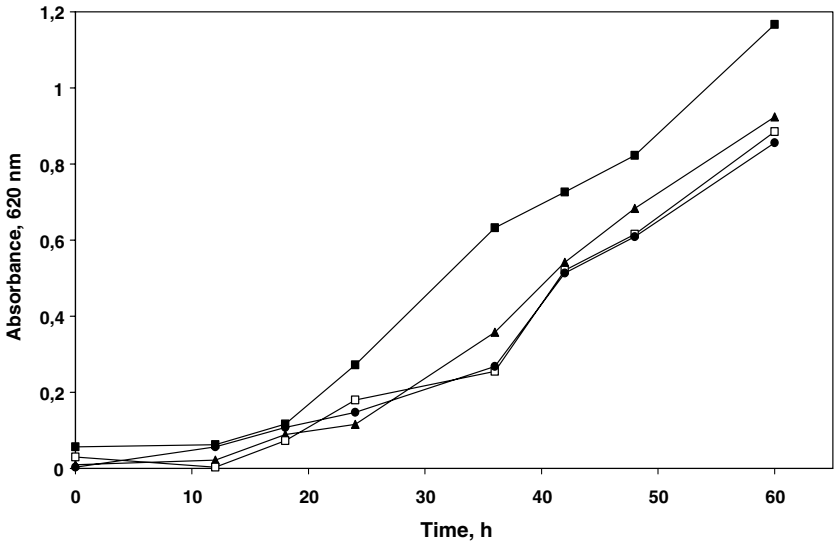

Figure 4. The effect of exudates from cowpea roots on the growth of *Fusarium oxysporum* fungi. The absorbance at 620 nm was taken as a measurement of fungal growth. Control (■); exudate obtained in phosphate buffer, pH 7.5 (▲), water, pH 6.0 (□) and acetate buffer, pH 4.5 (●). Experiments were run in triplicate and the standard errors were omitted for clarity (coefficients of variation were less than 20%).

protein demonstrates a positive reaction for the chitinase antiserum, confirming the presence of this enzyme in exudate from roots discharged during plant growth. These enzymes belong to the class of 'pathogenesis-related' proteins, which target major components of fungal cell walls, and have been shown to possess antifungal activity in several systems (Ji and Kué, 1996). These hydrolases may act on the tips of growing fungal hyphae where newly deposited chitin would be accessible to chitinases that are secreted by the plant (Nielsen et al., 1994; Radhajeyalakshmi et al., 2000).

Effect of root exuded proteins on fungal growth

To investigate cowpea root exudates for their antifungal functions, growth curves of the plant pathogen, *Fusarium oxysporum*, in the presence of the three root exudates were performed. After 60 h of fungal growth in the presence of exudates, an inhibitory effect on *F. oxysporum* growth was obtained as compared to the control medium (Figure 4). Photomicrographs of fungal mycelium were taken after the 60 h of growth period. Normal hyphal development (Figure 5a) was observed under control conditions. However, in the presence of the exudate fraction obtained in medium with acetate buffer (Figure 5b), there was a noticeable inhibition of conidial germination and hyphal development as compared with growth in the control medium. In this case, chitinases were suspected to affect the growth of this fungus in which chitin is a major structural polyssacharide. Growth inhibition by class I chitinases has been shown for *Tricoderma viridae*, a saprophytic fungus. However few fungi are sensitive to chitinase alone, while many more fungi are sensitive to a combination of chitinase and a β-1,3-Glucanase or in combination with other proteins or compounds that alter the membrane structure and permeability of the fungus (Datta and Muthukrishnan, 1999).

Recently, several defense proteins such as trypsin and papain inhibitors, glucan hydrolases and variant vicilins have been found in cowpea seeds, and these proteins are traditionally linked to plant defense mechanisms (Gomes et al., 1997; Xavier-Filho et al., 1996). In prior studies, two both a glucan-hydrolases, a chitinase of Mr 22,000 and a β-1,3-Glucanase of Mr 26,000, have been shown to inhibit the growth of several phytopathogenic fungi (Gomes et al., 1996). Recent results have also shown that peptides isolated from cowpea seeds exert detrimental effects on the development of the different phytopathogenic fungi and yeast (Carvalho et al., 2001). Seed and root exudates are the major driving force behind

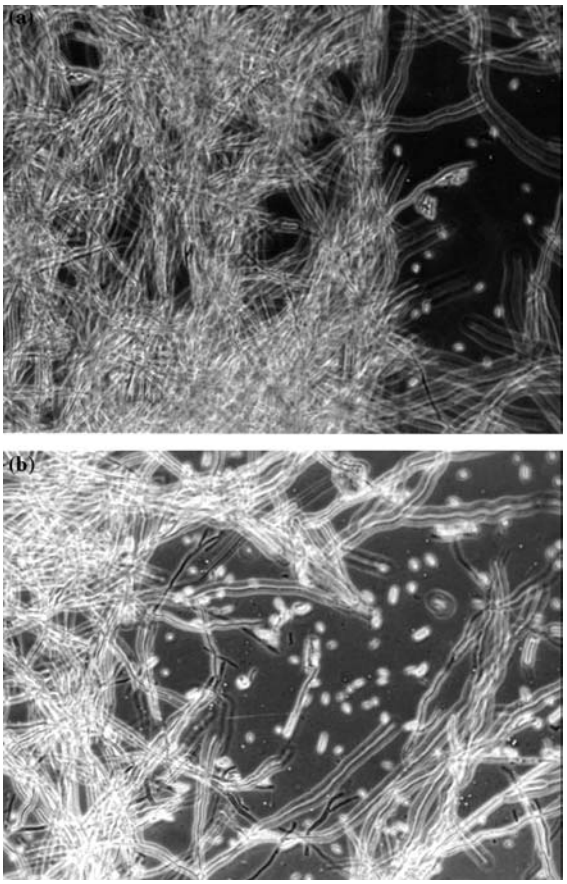

Figure 5. Light micrographs of *Fusarium oxysporum* mycelia after 60 h of fungal growth in control medium (a), and exudate obtained from roots in acetate buffer (b). Magnification bars = 15 μm.

the spermosphere and rhizosphere activities of soil-born plant pathogens, as well as associated microorganisms. Knowledge of when and how exudates initiate and regulate pathogen responses of plants is essential for an understanding of the interactions of soil-borne pathogens with seeds, roots and other microorganisms.

Tissue-specific localization of chitinase in cowpea roots

The spatial expression pattern of chitinase genes has been studied in a wide range of plant species including *Arabidopsis*, barley, broccoli, carrot, tobacco and maize. Many chitinases, like other plant defense proteins, are acid extractable, resistant to proteases, and are secreted extracellularly. These proteins can be either acidic or basic. The

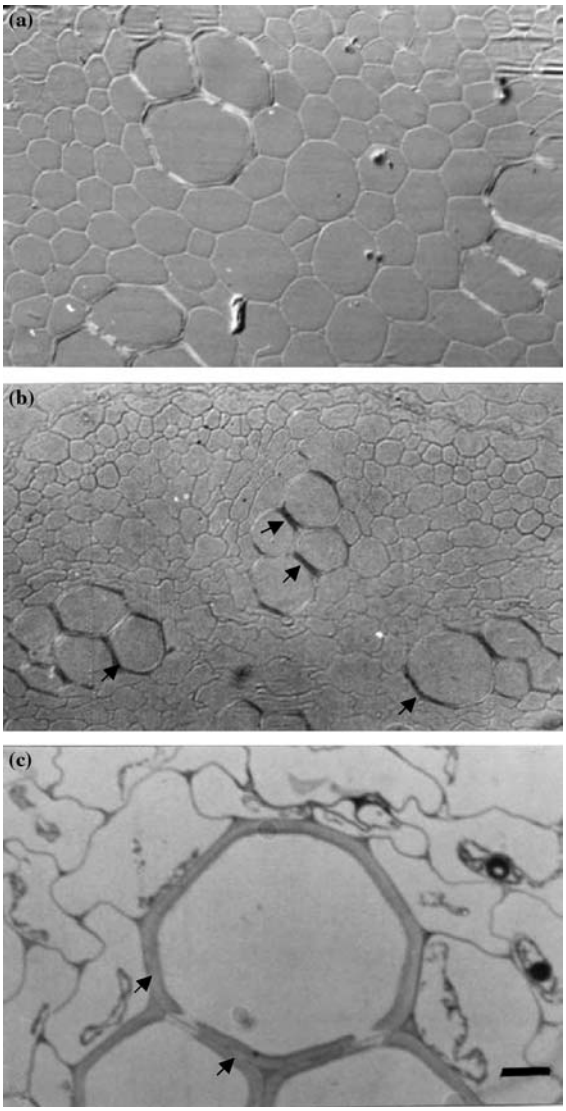

Figure 6. Immunolocalization of chitinase in sections from cowpea roots by optical microscopy using an anti-chitinase serum followed by treatment with 10 nm colloidal gold-conjugated secondary antibody and visualized by a silver intensification kit. The immunolabelling can be observed in the vessels elements cell wall of xylem (b and c). (a) corresponds to control sections in which the primary anti-serum was replaced by pre-immune serum. Bars = a, 20 μm; b, 40 μm and c, 8 μm.

acidic forms usually are secreted into the apoplast or extracellular environment, while the basic forms accumulate intracellularly in the vacuole (Punja and Zhang, 1993; Yeboah et al., 1998). The intracellular localization of class I chitinase, for example, depends on the presence or absence of a vacuolar targeting propeptide at the C-terminus of the precursor, which has been thoroughly

studied for the tobacco class I chitinase. Although there are some exceptions to this rule, it appears that a basic pI may be more appropriate for the vacuole and an acidic pI more appropriate for the cell wall. It certainly also hints at difference functions for both compartments in the defense mechanisms of plants (Datta and Muthukrishnan, 1999). In order to determine the spatial pattern of chitinase expression in cowpea roots, indirect immunolabelling was performed here to examine different parts of the roots. Chitinase was detected in the root hair zone. In addition, immunolabelling assays indicated that chitinase was localized in the xylem cell walls vessels elements (Figure 6) and in some endodermal cells. These results suggest that the apoplast is a possible pathway to transport these proteins through the root.

Trangenic plants have been produced to express chitinases in a constitutive manner. These plants were tested with several pathogenic fungi with negative results. However, bean class I chitinase protected tobacco against *Rhizoctonia solani*. Later, tobacco class I chitinase was found to reduce root colonization by the same fungi (Datta and Muthukrishnan, 1999). The identification of these proteins which are associated with defense mechanisms in cowpea is of importance for the establishment of appropriate techniques for their manipulation and general utilization through classical breeding techniques or through the more recent techniques employing genetic manipulation. Further studies will include the use of these mechanisms for the development of approaches to control fungal pathogens in this crop.

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